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### (54) Covalent attachment of specific binding members to a solid phase

Kovalente Kupplung von spezifischen Bindungspartnern an einer Festphase

Fixation covalente des membres de liaison spécifiques aux phases solides

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## Description

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

The present invention relates to methods and reagents for the covalent attachment of specific binding members to a solid support. In particular, the invention relates to the immobilization of specific binding members on solid supports for use in diagnostic assays and separation procedures.

#### 2. Description of Related Art

Binding assays have found widespread application in the field of clinical diagnostics for the detection and/or measurement of drugs, hormones, proteins, peptides, metabolites, microorganisms and other substances of interest, commonly referred to as analytes, in both biological and non-biological fluids. Binding assays incorporate specific binding members, typified by antibody and antigen immunoreactants, wherein one member of the specific binding pair is labeled with a signal-producing compound (e.g., an antibody labeled with an enzyme; a fluorescent compound; a chemiluminescent compound; a radioactive label; a direct visual label; etc.). For example, in a binding assay the test sample suspected of containing analyte can be mixed with a labeled anti-analyte antibody and incubated for the immunoreaction to occur. The reaction mixture is subsequently analyzed to detect either the label associated with an antibody/analyte complex (bound label) or the labeled antibody which is not complexed with analyte (free label), thereby enabling the detection or measurement of the analyte in the test sample.

Binding assays can be divided into two general categories known as homogeneous and heterogeneous assays. In the homogeneous assays, the signal produced by the bound label is different from the signal produced by the free label. As a result, bound and free label can be distinguished without physical separation of the individual reactants from the reaction mixture.

A well-known homogeneous binding assay is the enzyme-multiplied immunoassay technique (EMIT) which is disclosed in U.S. Patent 3,817,837. In the EMIT assay, the analyte present in the patient's test sample and an enzyme-labeled analyte compete for a limited amount of anti-analyte antibody. The specific binding of the antibody to the analyte-enzyme conjugate modulates the conjugate's enzymatic activity such that the enzyme activity is proportional to the amount of analyte in the test sample. Homogeneous binding assays have the advantages of being rapid, easy to perform, and readily adapted to automation. Their disadvantages are that they are susceptible to interferences caused by non-analyte substances in the test sample, they are generally limited to assays for low molecular weight analytes, and

they have a limited sensitivity.

In a heterogeneous binding assay, the signal produced by the bound label is indistinguishable from the signal produced by the free label; therefore, the free label and the bound label must be separated from one another to distinguish between their respective signals. In some cases, the complex with which the bound label is associated will substantially differ in molecular weight from the free labeled reactant so that centrifugation can be used to separate the heavier complex.

An alternative to centrifugation involves attaching at least one of the binding assay's reactants to a solid support. The solid support can then be separated from the test sample and the remaining assay reagents to provide for the separation of the free and bound label. The separation of the solid support and reaction mixture can be accomplished either by drawing-off the remaining reaction mixture or by physically removing the solid phase from the reaction mixture. The solid support can also be treated or washed to remove interfering substances prior to the detection or measurement of the label associated with the solid phase.

Longer incubation times may be required in the heterogeneous assay. This is because the kinetics of a reaction between a solid phase-bound specific binding member and its complementary binding partner tend to be slower than the kinetics of the same reaction when both binding members are in solution. The heterogeneous assays, however, are in general more sensitive than homogeneous assays and less prone to interferences, because interfering substances can be removed by the wash steps.

Variations to this general solid phase separation scheme have been developed, but they typically involve the binding of the analyte to a specific binding member which is attached to a solid phase. Generally, specific binding members are attached to or immobilized on the solid phase by adsorption or covalent bonding. Adsorption results from the action of the solid phase in attracting and holding the specific binding member. With covalent bonding, the specific binding member and the solid phase are chemically reacted to result in a bond which immobilizes the specific binding member on the solid phase.

The linkage between the solid phase and the immobilized specific binding member can greatly affect the binding of that specific binding member to the analyte. For example, antibodies have extremely specific structural, spatial and polar configurations which enable them to recognize and bind to a specific analyte (e.g., antigen). When antibodies are used in an assay for the detection of antigens, the antibodies may be the specific binding members linked to the solid phase. The proximity of the solid phase to the antibody, however, can partially or completely block the sites on the antibody to which the antigen binds. In addition, the linkage between the antibody and the solid phase can alter the conformation of the antibody and thereby affect the an-

libody's ability to bind to the analyte. The same limitations hold for the linkage of other specific binding members to a solid phase; the specific binding members may attach in a spectrum of positions ranging from complete steric hindrance of the binding site to unhindered access, and/or the conformation of the specific binding member can change upon linking to the solid phase so that its complementary binding partner can no longer recognize it or bind to it. As may be expected, the sensitivity of the assay declines with increasing levels of steric hindrance and loss of reactivity.

A conventional method for covalently attaching a proteinaceous specific binding member to a polymeric solid phase involves the use of carbodiimide to crosslink the amine groups of the protein to the carboxyl groups on the surface of the solid phase. Alternatively, glutaraldehyde is used to crosslink the amines of the protein to surface bound amines on the solid phase. These crosslinking methods, however, are poorly controlled, often resulting in protein/protein crosslinking and non-specific reactions, such as the over-modification of the protein which may result in a decrease in the binding capacity of the specific binding member. In addition, the protein that is so immobilized has poor reactivity, making it necessary to bind large amounts of the protein to the solid phase to obtain suitable assay sensitivity.

There have been two main approaches to solving the crosslinking problems associated with the heterogeneous assays. One approach has been to complete the reaction of the binding partners prior to the immobilization of the newly formed complex upon the solid phase. The other technique has been to extend the length of the linkage between the specific binding member and the solid phase. The linking or coupling agent must maintain the linkage during the chemical manipulations of the assay, as well as during the physical manipulations of washing and separation steps. Extended length heterobifunctional coupling agents have been described in EP-A-314,127 (Abbott Laboratories), wherein the covalent attachment of specific binding members to the solid phase is accomplished using an extended length molecular chain having at least one linking group that is reactive with a chemical group on the solid phase and at least one linking group that is reactive with a chemical group on the specific binding member.

Patent Abstracts of Japan, vol. 5, no. 8, (C-39)(680) discloses specific binding members attached to insoluble carriers through two disulfide linkages and an interlying spacer group.

US-A-4 529 712 discloses conjugates of an animal cell and a specific binding member having an amino group, said specific binding member being connected to the amino-reactive end of said cross-linking reagent via an amide bond and said cell being connected to the thiol-reactive end of said cross-linking reagent by way of a thiol group on the surface of said cells or by way of thiol groups derived from amino groups on the surface of said cells, said surface amino groups having been

converted into thiol group-containing amides.

Johnson, J. C., "Immobilized Enzymes, Preparation and Engineering, Recent Advances", 1979, Noyes Data Corporation, Park Ride, New Jersey, USA, pages 258-260 summarizes the contents of US-A-3 904 478 which discloses thio-derivatives of coenzymes, such as thioether derivatives, which may be coupled to an amino group, for example, on a bifunctional organic compound, said compound being also coupled to a support material.

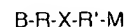
The reaction between a nucleophile such as an amine and a thioether results in the displacement of the thioether group as illustrated by Leonard, E. O., et al., Archives of Biochemistry and Biophysics, 99: 16-24 (1962).

Carlsson, J., et al., Biochem. J. 173: 723-737 (1978) discusses the reversibility of disulfide bonds and states that disulfide bridges between protein molecules are easily split by reduction or thiol-disulfide exchange.

## SUMMARY OF THE INVENTION

The present invention provides methods for preparing thiolated solid phase materials and immobilized specific binding members.

A reactive solid phase, having an immobilized specific binding member, produced by the present invention, comprises a compound of the formula:



wherein B is a solid phase having a reactive member selected from the group consisting of amino, carboxyl and thiol groups;

M is a specific binding member having an amino group;

R and R' are coupling agents independently selected from the group consisting of heterobifunctional and homobifunctional reagents; and

X is a dithio compound linked to R through a thioether and to R' through a thioether; wherein R is attached to B via the amino, carboxyl or thiol group of B, and R' is attached to M via the amino group of M.

The immobilized specific binding member is prepared by first reacting the solid phase with a first coupling agent, thereby forming a solid phase/coupling agent complex. The aminated specific binding member is reacted with a second coupling agent, thereby forming a specific binding member/coupling agent complex. The solid phase/coupling agent complex is then reacted with a dithiol compound to form a thiolated solid phase/coupling agent complex. And, the thiolated solid phase/coupling agent complex is reacted with the specific binding member/coupling agent complex to form the dithioether crosslinked solid phase/specific binding member com-

plex. Alternatively, the specific binding member/coupling agent complex can be reacted with the dithiol compound to form a thiolated specific binding member/coupling agent complex, which is then reacted with the solid phase/coupling agent complex to form the immobilized specific binding member.

The present invention is also of use in forming a thiolated solid phase represented by the formula:



wherein B is a solid phase having an amino, carboxyl or thiol group, R is a heterobifunctional or homobifunctional coupling agent and XH is a dithio compound linked to R through a thioether. The thiolated solid phase is made by reacting the solid phase with a heterobifunctional or homobifunctional coupling agent to form a solid phase/coupling agent complex and then reacting the complex with a dithiol compound to form a thiolated solid phase/coupling agent complex.

The resulting immobilized specific binding member can be used in diagnostic binding assays. And, the immobilized specific binding member can optionally be incorporated into or onto a support medium.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a diagrammatical illustration of the modification of an amino-bearing specific binding member to include a thiol-reactive group using a maleimido-NHS active ester coupling agent.

Fig. 2 is a diagrammatical illustration of the modification of an amino-bearing specific binding member to include a thiol-reactive group using an active halogen-NHS active ester coupling agent.

Fig. 3 is a diagrammatical illustration of the modification of an amino-bearing solid phase with a dithiol compound to form a thiolated solid phase.

Fig. 4 is a diagrammatical illustration of the sulphydryl crosslinking of a thiolated solid phase/coupling agent complex and a specific binding member/coupling agent complex to form an immobilized specific binding member.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention provides novel methods for the covalent linkage of the specific binding member and the solid phase, such as the immobilization of a protein on a solid phase for use in an immunoassay. The method can be used to covalently crosslink two amino-containing components. These components include, but are not limited to, antibodies, enzymes, peptides, cells, haptens, small molecules, solid phases, liposomes, and polymers. The general methodology of the present invention involves the modification of the specific binding member and/or the solid phase to incorporate thiol-re-

active functional groups. The activated specific binding member is then allowed to react with the thiolated solid phase to produce the covalent linkage. Both the solid phase and the specific binding member are individually modified to include extended length homobifunctional or heterobifunctional coupling agents, which in turn can be crosslinked through a dithiol compound.

The present invention provides greater control over the chemistry for the production of both derivatized solid supports and immobilized specific binding members. The invention enables the production of immobilized specific binding members which have increased sensitivity, specificity and stability, while requiring less specific binding member be used. In addition, the present invention may alter the surface charge of the solid phase, which in some instances eliminates the charge-related nonspecific interactions which can occur in binding assays. The covalently bound specific binding member/solid phase compounds of the present invention can be used in both sandwich and competitive heterogeneous binding assays, in both forward and reverse assay techniques.

#### Definitions

The following definitions are applicable to the present invention.

The term "specific binding member", as used herein, refers to a member of a specific binding pair, i.e., two different molecules where one of the molecules through chemical or physical means specifically binds to the second molecule. In addition to antigen and antibody-specific binding pairs, other specific binding pairs include biotin and avidin, carbohydrates and lectins, complementary nucleotide sequences (including probe and capture nucleic acid sequences used in DNA hybridization assays to detect a target nucleic acid sequence), complementary peptide sequences including those formed by recombinant methods, effector and receptor molecules, enzyme cofactors and enzymes, enzyme inhibitors and enzymes, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding member. For example, a derivative or fragment of the analyte, i.e., an analyte-analog, can be used so long as it has at least one epitope in common with the analyte. Immunoreactive specific binding members include antigens, haptens, antibodies, and complexes thereof including those formed by recombinant DNA methods or peptide synthesis.

"Analyte", as used herein, is the substance to be detected in or separated from the test sample using the present invention. The analyte can be any substance for which there exists a naturally occurring specific binding member or for which a specific binding member can be prepared. In addition, the analyte may bind to more than one specific binding member. "Analyte" also includes any antigenic substances, haptens, antibodies, and

combinations thereof. The analyte can include a protein, a peptide, an amino acid, a hormone, a steroid, a vitamin, a drug including those administered for therapeutic purposes as well as those administered for illicit purposes, a bacterium, a virus, and metabolites of or antibodies to any of the above substances.

"Solid phase", as used herein, includes the material upon which a specific binding member can be immobilized for use in diagnostic assays, affinity chromatography and separation procedures. While the Examples which follow generally deal with microparticle solid phases made of polymeric materials, many other solid phase configurations are possible so long as the material includes or can be formed or derivatized to accept the requisite linking groups enabling the immobilization of a specific binding member.

The solid phase can include, without limitation, polymeric or glass beads, microparticles, tubes, sheets, plates, slides, wells, tapes, test tubes, or the like. Natural, synthetic or naturally occurring materials that are synthetically modified, can be used as the solid phase including polysaccharides, e.g., cellulose materials such as paper and cellulose derivatives such as cellulose acetate and nitrocellulose; silica; silicon particles; inorganic materials such as deactivated alumina, or other inorganic finely divided material uniformly dispersed in a porous polymer matrix, with polymers such as vinyl chloride, vinyl chloride polymer with propylene, and vinyl chloride polymer with vinyl acetate; polyethylene; cloth, both naturally occurring (e.g., cotton) and synthetic (e.g., nylon); porous gels such as silica gel; polymeric films such as polyacrylates; and the like. It will be readily apparent to those skilled in the art that a variety of solid phase materials can be adapted for use in accordance with the present invention.

In addition, a solid phase prepared in accordance with the present invention can be incorporated within or upon a separate support medium. Support media include any suitable absorbent, chromatographic, bibulous, porous or capillary material. For example, the support media can include a fiberglass, cellulose or nylon pad for use in a flow-through assay device as described in co-owned and copending European Patent Publication No. 217,403 published April 8, 1987. The device disclosed therein comprises a substantially planar layer of material having a porous matrix of fibers, upon which are immobilized a plurality of substantially spherical microparticles bearing an immobilized specific binding member. Similarly, a dipstick for a dip and read assay or a test strip for chromatographic (e.g., paper or glass fiber) or thin layer chromatographic (e.g., nitrocellulose) techniques can be used.

The term "test sample", as used herein, includes a naturally occurring or artificially formed liquid test medium suspected of containing the analyte of interest. In diagnostic assays, the test sample is generally a biological fluid or a dilution thereof. Biological fluids from which an analyte can be determined include serum, whole

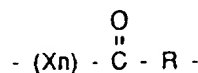
blood, plasma, urine, saliva, amniotic and cerebrospinal fluids, and the like. The reagents and methods of the present invention can also be designed to determine food product and environmental analytes of interest.

The term "coupling agent", as used herein, includes bifunctional crosslinking or coupling agents, i.e., molecules containing two reactive groups or "ends", which may be tethered by a spacer. The reactive ends can be any of a variety of functionalities including, but not limited to: amino reacting ends such as *n*-hydroxysuccinimide (NHS) active esters, imidoesters, aldehydes, epoxides, sulfonyl halides, isocyanate, isothiocyanate, and nitroaryl halides; and thiol reacting ends such as pyridyl disulfides, maleimides, thiophthalimides, and active halogens. The heterobifunctional crosslinking reagents have two different reactive ends, e.g., an amino-reactive end and a carboxyl-reactive end, while homobifunctional reagents have two similar reactive ends.

Commercially available heterobifunctional reagents for use in the present invention include, but are not limited to, the maleimido-NHS active esters coupling agents such as *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS); succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC); succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB) and derivatives thereof, including sulfosuccinimidyl derivatives such as sulfosuccinimidyl-4-(*N*-maleimido-methyl) cyclohexane-1-carboxylate (sulfo-SMCC); *m*-maleimidobenzoyl-sulfosuccinimide ester (sulfo-MBS) and sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate (sulfo-SMPB).

Other heterobifunctional reagents include the commercially available active halogen-NHS active esters coupling agents such as *N*-succinimidyl bromoacetate and *N*-succinimidyl (4-iodoacetyl)aminobenzoate (SI-AB) and the sulfosuccinimidyl derivatives such as sulfosuccinimidyl (4-iodoacetyl)aminobenzoate (sulfo-SI-AB).

Yet another group of coupling agents includes the extended length heterobifunctional coupling agents described in Application EP-A-314,127. The extended length heterobifunctional coupling agents include maleimido-NHS active ester reagents wherein the spacer is represented by the formula:



wherein the X is a substituted or unsubstituted amino acid, having from three to ten carbon atoms in a straight chain; *n* is from one to ten; and R is an alkyl, cycloalkyl, alkyl-cycloalkyl or an aromatic carboxylic ring. The term alkyl-cycloalkyl includes alkyl groups linked to cycloalkyl ring structures where the alkyl group links the cycloalkyl to a maleimide or carbonyl group. The term alkyl includes straight or branched alkyl groups, preferably low

er alkyl groups having from one to six carbon atoms.

If a spacer is present to tether the two reactive groups of the coupling agent, the spacer can be any molecular chain that is non-reactive, stable and non-binding to the analyte or other specific binding members with which it will be used. The length of the spacer can be varied and can range from the size of a single atom to the sizes disclosed in EP-A-314,127 or larger.

The choice of a coupling agent depends upon the acceptable performance of that particular agent with the particular solid phase and specific binding member to be used in the diagnostic assay. Therefore, it will be appreciated by those skilled in the art that the coupling agent used in a given assay will generally be determined empirically. In addition, the specific binding member will include one or more amino, carboxyl or thiol groups or can be derivatized by the incorporation of an amino, carboxyl or thiol group thereby enabling the reaction of the specific binding member with a coupling agent. "Activated species" refer to specific binding members and solid phase materials which contain a reactive group through the incorporation of a coupling agent, for example, the activation of a protein with sulfo-MBS. Proteinaceous specific binding members with cysteine residues at the protein's active site can have their activity decreased by the addition of a coupling agent, therefore the cysteine residues in the active site must be protected, by means known in the art, prior to reacting the protein with the coupling agent.

#### Preparation of activated or derivatized solid phases and immobilized specific binding members

The general methodology of the present invention involves the modification of a solid phase by the introduction of thiol groups. A specific binding member, e.g., a protein antigen, is also modified to contain thiol-reactive functional moieties such as maleimides or active halogens.

Two methods by which a specific binding member bearing an amino group can be modified to contain a thiol-reactive functional group are diagrammatically illustrated in Figures 1 and 2. In particular, Figure 1 illustrates the activation of a protein specific binding member through the use of a maleimido-NHS active ester heterobifunctional reagent to incorporate a thiol-reactive group on the protein. The R in the coupling agent represents alkyl, cycloalkyl, alkyl-cycloalkyl or an aromatic carboxylic ring as described above. The Z in the coupling agent typically represents a hydrogen atom, or optionally represents an inert polar group such as SO<sub>3</sub> which imparts the characteristic of water solubility upon the coupling agent.

Referring to Figure 2, the specific binding member is reacted with an active halogen-NHS active ester heterobifunctional reagent, wherein R and Z are defined as described above.

Figure 3 represents the reaction of an aminated sol-

id phase with an active halogen-NHS active ester heterobifunctional reagent, wherein R and Z are defined as described above and I is a sample halogen. The resultant solid phase/coupling agent complex is then activated by reacting the complex with a thiol introducing agent, e.g., a dithiol compound represented by HS-X-SH, to produce a thiolated solid phase. As with the preparation of the specific binding member/coupling agent complex in Fig. 1, a thiolated solid phase/coupling agent complex can also be prepared through the use of a maleimido-NHS active ester heterobifunctional reagent.

Both the solid phase and the specific binding member can be modified to include a coupling agent, the coupling agents providing the required amino, thiol or carboxyl groups. One of the modified components, such as the solid phase/coupling agent complex, is then treated with a dithiol compound to introduce sulfhydryl groups to the component thereby forming a thiolated solid phase/coupling agent complex. Finally, the specific binding member/coupling agent complex is added to the thiolated solid phase/coupling agent complex to form a crosslinked product.

An example of the sulfhydryl linkage of a specific binding member/coupling agent complex and a solid phase/coupling agent complex is diagrammatically illustrated in Figure 4. An aminated microparticle is represented in the upper left portion of the illustration. The solid phase microparticles include commercially available latex microparticles bearing amino groups, as well as microparticles which have been modified or activated to contain amino groups. The microparticles are mixed with a heterobifunctional coupling agent, e.g., sulfo-MBS, to produce a microparticle/coupling agent complex. The microparticle/coupling agent complex is then reacted with a dithiol compound, e.g., dithiothreitol (DTT), to produce a thiolated microparticle/coupling agent complex. An aminated protein, such as an amine group bearing antigen, is represented in the upper left portion of the illustration. The protein is also linked to a heterobifunctional coupling agent to produce a protein/coupling agent complex. Lastly, the thiolated microparticle/coupling agent complex and the protein/coupling agent complex are reacted to produce a dithioether crosslinked microparticle and protein, i.e., an immobilized specific binding member.

#### EXAMPLES

The following examples describe methods for synthesizing the novel activated solid phase products (i.e., the thiolated solid phase/coupling agent complex) and the immobilized specific binding member products of present invention.

## I. Protein Immobilization Through Dithioether Linkages

### Example 1

#### Preparation of Recombinant HIV-1 gp-41 Derivatized Microparticles

##### a) Preparation of Thiolated Microparticles

A 3.0 ml suspension of amino-microparticles (3 $\mu$  diameter, 2.5% solids, Polysciences, Inc., Warrington, PA) was placed into a 15 ml polypropylene centrifuge tube and centrifuged at 3000 rpm for 10 minutes. The supernatant was decanted and discarded. The microparticle pellet was resuspended in 3.0 ml of 10 mM phosphate buffered saline, pH 7.2 (PBS) by vortexing until the suspension was homogeneous. A 0.3 ml solution of N-maleimidobenzoyl-N-hydroxysulfo-succinimide ester (sulfo-MBS, Pierce Chemical Co., Rockford, IL), 10 mg per ml PBS, was added to the microparticle suspension, and the mixture was allowed to react for 1 hour, at room temperature (20-25°C) with end-over-end rotation. The mixture was centrifuged, and the supernatant was decanted and discarded. The maleimido-microparticle pellet was washed by resuspension in 10 ml of PBS with vortexing, centrifuging, and then decanting the supernatant. The washing process was performed three times. The microparticle pellet was then resuspended in a 10 ml solution of 0.1M dithiothreitol in PBS. The microparticle suspension was mixed at room temperature by end-over-end rotation for 1 hour. The suspension was centrifuged, and the supernatant was decanted and discarded. The thiolated microparticles were resuspended in a 10 ml solution of 0.1% (v/v) of Tween® -20 in PBS, by vortexing, to achieve a homogeneous suspension. The mixture was then centrifuged, and the supernatant was discarded. The resuspension in 0.1% Tween® -20/PBS, centrifugation, and supernatant decanting steps were repeated for three additional cycles. The thiolated microparticle pellet was then resuspended with PBS to a final volume of 3.0 ml. The thiolated microparticles reacted optimally when used within 3 hours of removing the dithiothreitol.

##### b) Activation of Recombinant HIV-1 gp41 with sulfo-MBS

A 1.2 ml solution of 0.3 mg protein in 1.8% sodium dodecyl sulfate (SDS)/PBS (w/v) was placed in a reaction vessel. The protein specific binding member was an HIV antigen. Exemplary HIV proteins include the recombinant protein constructs such as p24 and gp41 sequences known in the art. A 1.0 ml solution of 30% Tween® -20 in PBS (v/v) was added, and the solution mixed, followed by the addition of 20  $\mu$ g sulfo-MBS in 0.8 ml PBS to produce a specific binding member/coupling agent complex. The solution was again mixed and

allowed to set at room temperature for 1 hour.

##### c) Covalent Linking of Maleimidobenzoyl Activated HIV-1 gp41 to Thiolated Microparticles

The activated protein (3.0 ml from Example 1. b) was combined with the suspension of thiolated microparticles (3.0 ml from Example 1. a). The mixture was allowed to react at room temperature overnight (14 to 18 hours) while rotating end-over-end. The protein coated microparticle suspension was centrifuged, as described in Example 1. a, and the supernatant was decanted and discarded. The pellet was resuspended in 10 ml of 10 mM 2-mercaptoethanol by vortexing, and the mixture was then rotated end-over-end for 1 hour. The suspension was centrifuged and the supernatant discarded. The microparticle pellet was washed by 4 cycles of resuspension in 10 ml of 0.1% Tween® -20 in PBS (v/v), centrifugation, and then decanting the supernatant. The particles were then resuspended in PBS with 0.1% sodium azide (w/v) to 10 ml of a 0.75% solids suspension. The immobilized specific binding member preparation was stored at 4 to 8°C.

### Example 2

#### Preparation of Recombinant HIV-1 p24 Coated Microparticles

##### a) Activation of Recombinant HIV-1 p24 with sulfo-MBS

A solution of 375  $\mu$ g p24 in 0.5 ml of 0.5% SDS/PBS (w/v) was heated at 45°C for 20-30 minutes. After heating, additions of 0.5 ml of 30% Tween® -20 in PBS (v/v) and 0.5 ml of PBS containing 60  $\mu$ g sulfo-MBS were made to the protein solution. The reaction mixture was vortexed and set at room temperature for 1 hour to produce the specific binding member/coupling agent complex.

##### b) Covalent Coupling of Derivatized p24 to Thiolated Microparticles

A suspension of 3.0 ml of thiolated microparticles (as described in Example 1. a) was centrifuged and the supernatant discarded. The pellet was resuspended in the activated p24 solution with vortexing, and was mixed by end-over-end rotation at 20-25°C for 14-18 hours. The suspension was then centrifuged, and the supernatant discarded. The p24 coated microparticle pellet was resuspended in 10 ml of 10 mM 2-mercaptoethanol and mixed by end-over-end rotation at 20-25°C for 1 hour. The suspension was again centrifuged and the supernatant discarded. The pellet was then washed with 4 cycles of resuspension in 10 ml of 0.1% Tween® -20 in PBS (v/v), centrifugation, and discarding the supernatant. The pellet was resuspended in PBS containing 0.1% sodium azide (w/v) to a volume of 10 ml, and the

immobilized specific binding member was stored at 4-8°C.

#### Example 3

##### Preparation of Recombinant HIV Protein-Coated Microparticles Using Urea Activation of the Protein

##### a) Activation of Recombinant HIV Protein with sulfo-MBS

A solution of 600 µg of HIV protein in 55 µl of 8M urea was placed in an appropriate vessel. To this, 40 µg of sulfo-MBS in 40 µl of 8M urea were added. The solution was thoroughly mixed and set at room temperature for 1 hour.

##### b) Covalent Coupling of Recombinant HIV Protein to Thiolated Microparticles

Activated protein, from Example 3. a, was added to a 3.0 ml suspension of thiolated microparticles from Example 1. a. The suspension was incubated at 20-25°C for 14-18 hours with end-over-end rotation. The protein coated microparticles were then centrifuged, treated with 2-mercaptoethanol, washed, resuspended, and stored substantially in accordance with the procedure described in Example 1. c.

#### II. Protein Immobilization Through Dithioether Linkages Using Alternate Heterobifunctional Reagents in Microparticle Thiolation

#### Example 4

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using sulfo-SMCC in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 1 using sulfo-SMCC coupling agent (Pierce) instead of sulfo-MBS in the preparation of the thiolated microparticles.

#### Example 5

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using sulfo-SIAB in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 1 using sulfo-SIAB (Pierce) instead of sulfo-MBS in the preparation of thiolated microparticles.

#### Example 6

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using SIAB in Microparticle Thiolation

A 3.0 ml suspension of amino-microparticles was centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and discarded. The pellet was resuspended in 3.0 ml of a 1:1 mixture of PBS and dimethylsulfoxide (DMSO) by vortexing. A 0.3 ml solution containing 3.0 mg SIAB (Pierce) in DMSO was added to the suspension which was then mixed by end-over-end rotation at 20-25°C for one hour. The suspension was centrifuged and the supernatant discarded. The iodoacetyl microparticles were washed using 3 cycles of resuspension in 10 ml of PBS, centrifugation, and removal of the supernatant. The pellet was then resuspended in a 10 ml solution of 0.1 M dithiothreitol in PBS. The suspension was mixed for one hour at 20-25°C, then centrifuged and the supernatant discarded. The thiolated microparticles were washed as described above using 4 cycles with 10 ml of 0.1% Tween® -20 in PBS (v/v) in each cycle. The particles were then resuspended in 10 ml of PBS. The preparation of the protein/coupling agent complex and its crosslinking to the thiolated microparticles were repeated substantially in accordance with the procedures of Example 1. b and c.

#### Example 7

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using SMCC in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 6 using SMCC (Pierce) instead of SIAB in the preparation of thiolated microparticles.

#### Example 8

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using a 16 Atom Heterobifunctional Linker Group in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 6 using the 16 atom maleimido-n-hydroxysuccinimidyl active ester compound described in EP-A-314,127 instead of SIAB.

#### Example 9

##### Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using a 23 Atom Heterobifunctional Linker Group in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol de-



scribed in Example 6 using the 23 atom maleimido-n-hydroxysuccinimidyl active ester compound described in EP-A-314,127 instead of SIAB.

#### Example 10

Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using a 30 Atom Heterobifunctional Linker Group in Microparticle Thiolation

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 6 using the 30 atom maleimido-n-hydroxysuccinimidyl active ester compound described in EP-A-314,127 instead of SIAB.

### III. Protein Immobilization Through Dithioether Linkages Using Alternate Heterobifunctional Reagents in Protein Activation

#### Example 11

Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using sulfo-SMCC Activated gp41

The immobilized specific binding member was made substantially in accordance with the protocol of Example 1 using sulfo-SMCC instead of sulfo-MBS in the activation of recombinant gp41 described in Example 1. b.

#### Example 12

Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using sulfo-SIAB Activated gp41

The immobilized specific binding member was made substantially in accordance with the protocol of Example 1 using sulfo-SIAB instead of sulfo-MBS in the activation of recombinant gp41 described in Example 1. b.

#### Example 13

Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using SMCC Activated gp41

A 1.0 ml solution of 0.3 mg protein in PBS was mixed with 2.0 ml of DMSO containing 20 µg of SMCC. The solution was mixed and set at 20-25°C for 1 hour to produce the specific binding member/coupling agent complex. The preparation of thiolated microparticles and the protein-to-microparticle coupling procedures were repeated as described in Example 1 a and c, respectively.

#### Example 14

Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using SIAB Activated gp41

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 13 using SIAB instead of SMCC in the derivatization of recombinant gp41.

#### Example 15

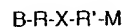
Preparation of Recombinant HIV-1 gp41 Derivatized Microparticles Using a 30 Atom Heterobifunctional Linker Modified gp41

The immobilized specific binding member was made substantially in accordance with the protocol described in Example 13 using the 30 atom maleimido-n-hydroxysuccinimidyl active ester compound, described in EP-A-314,127 instead of SMCC in the activation of recombinant gp41.

It will be appreciated by one skilled in the art that the concepts of the present invention are equally applicable to the crosslinking of many specific binding members, solid phase materials and coupling agents other than those exemplified above. The embodiments described herein are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments described in detail, but it is intended to encompass all equivalents and subject matter within the scope of the invention as set forth in the following claims.

### Claims

1. A reactive solid phase, having an immobilized specific binding member, comprising a compound of the formula:



wherein B is a solid phase having a reactive member selected from the group consisting of amino, carboxyl and thiol groups; M is a specific binding member having an amino group; R and R' are coupling agents independently selected from the group consisting of heterobifunctional and homobifunctional reagents; and X is a dithio compound linked to R through a thioether and to R' through a thioether; wherein R is attached to B via the amino, carboxyl or thiol group of B, and R' is attached to M via the amino group of M.

2. A reactive solid phase having a reactive thiol, comprising a compound of the formula:



wherein B is a solid phase having a reactive member selected from the group consisting of amino, carboxyl, and thiol groups;

R is a coupling agent selected from the group consisting of heterobifunctional and homobifunctional reagents; and

XH is a dithio compound linked to R wherein one thio function forms a thioether with R and the other thio function is the reactive thiol.

3. The reactive solid phase according to Claim 1 or 2, wherein said solid phase is selected from the group consisting of polymeric beads, microparticles, tubes, sheets, plates, slides, wells and tapes.

4. The reactive solid phase member according to Claim 1, wherein R and R' are heterobifunctional coupling agents, both of which are the same.

5. The immobilized specific binding member according to Claim 1, wherein said specific binding member is one member of an immunoreactive specific binding pair.

6. The reactive solid phase member according to Claim 1 or 2, further comprising a support medium.

7. The immobilized specific binding member according to Claim 1, wherein said solid phase is a polymeric microparticle and wherein said specific binding member is an antigen.

8. The immobilized specific binding member according to Claim 7, wherein said solid phase is an amino microparticle and wherein said specific binding member is an HIV antigen.

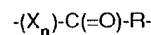
9. The immobilized specific binding member according to Claim 1, wherein said specific binding member is selected from the group consisting of a polynucleotide and a polypeptide.

10. The reactive solid phase member according to Claim 1 or 2, wherein said coupling agent(s) are independently selected from the group consisting of maleimido-N-hydroxysuccinimide active esters and active halogen-N-hydroxysuccinimide active esters.

11. The reactive solid phase according to Claim 10, wherein at least one coupling agent is a maleimido-N-hydroxysuccinimide ester selected from the

group consisting of *m*-maleimidobenzoyl-N-hydroxy-succinimide ester; succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; succinimidyl 4-(*p*-maleimidophenyl)butyrate; *m*-maleimido-benzoyl-sulfosuccinimide ester; and sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate.

12. The reactive solid phase according to Claim 10, wherein at least one coupling agent is a maleimido-N-hydroxysuccinimide active ester having a spacer represented by the formula:



wherein X is a substituted or unsubstituted amino acid having from three to ten carbon atoms in a straight chain;

n is from one to ten; and

R is an alkyl, cycloalkyl, alkyl-cycloalkyl or an aromatic carboxylic ring.

13. The immobilized specific binding member according to Claim 10, wherein at least one coupling agent is an active halogen-N-hydroxysuccinimide active ester selected from the group consisting of N-succinimidyl bromoacetate, N-succinimidyl (4-iodoacetyl)aminobenzoate and sulfosuccinimidyl (4-iodoacetyl)aminobenzoate.

14. A method for producing an immobilized specific binding member, comprising the steps of:

a. reacting a solid phase, having a reactive member selected from the group consisting of amino, carboxyl and thiol groups, with a first coupling agent selected from the group consisting of heterobifunctional and homobifunctional reagents having a first function reactive with the reactive member of said solid phase and a second function reactive with a thiol, thereby forming a solid phase/coupling agent complex by reaction between said reactive member and first function of the first coupling agent;

b. reacting a specific binding member having an amino group with a second coupling agent having a first function reactive with said amino group and a second function reactive with a thiol, thereby forming a specific binding member/coupling agent complex by reaction between said amino group and first function of the second coupling agent; and

c. reacting said solid phase/coupling agent complex and said specific binding member/coupling agent complex with a dithiol compound thereby forming a solid phase/specific binding member complex wherein the solid phase and the specific binding member are

coupled together through two thioethers.

15. A method for producing a thiolated solid phase, comprising the steps of:

a. reacting a solid phase, having a reactive member selected from the group consisting of amino, carboxyl and thiol groups, with a coupling agent selected from the group consisting of heterobifunctional and homobifunctional reagents, thereby forming a solid phase/coupling agent complex; and  
b. reacting said solid phase/coupling agent complex with a dithiol compound wherein one thiol group links said dithiol compound to said solid phase/coupling agent complex through a thioether and the other thiol group is the reactive thiol.

16. The method of Claim 14 or 15, wherein said solid phase is selected from the group consisting of polymeric beads, microparticles, tubes, sheets, plates, slides, wells and tapes.

17. The method according to Claim 14 or 15, wherein said coupling agent(s) are heterobifunctional reagents.

18. The method according to Claim 14, further comprising incorporating said crosslinked solid phase/specific binding member complex upon or within a support medium.

19. The method according to Claim 14, wherein said specific binding member is one member of an immunoreactive specific binding pair.

20. The method according to Claim 19, wherein said solid phase is a polymeric microparticle and wherein said specific binding member is an antigen.

21. The method according to Claim 20, wherein said solid phase is an amino microparticle and wherein said specific binding member is an HIV antigen.

22. The method according to Claim 14, wherein said specific binding member is selected from the group consisting of a polynucleotide and a polypeptide.

23. The method according to Claim 14, wherein said solid phase/coupling agent complex is reacted with the dithiol compound, to form a thiolated solid phase/coupling agent complex, and then the specific binding member/coupling agent complex is reacted with said thiolated solid phase/coupling agent complex.

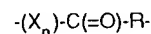
24. The method according to Claim 14, wherein said

specific binding member/coupling agent complex is reacted with the dithiol compound to form a thiolated specific binding member/coupling agent complex and then the solid phase/coupling agent complex is reacted with said thiolated specific binding member/coupling agent complex.

25. The method according to Claim 14 or 15, wherein said coupling agent(s) are independently selected from the group consisting of maleimido-N-hydroxysuccinimide active esters and active halogen-N-hydroxysuccinimide active esters.

26. The method according to Claim 25, wherein at least one coupling agent is a maleimido-N-hydroxysuccinimide ester selected from the group consisting of *m*-maleimidobenzoyl-N-hydroxy-succinimide ester; succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate; succinimidyl 4-(*p*-maleimidophenyl)butyrate; *m*-maleimidobenzoyl-sulfosuccinimide ester; and sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate.

27. The method according to Claim 25, wherein at least one coupling agent is a maleimido-N-hydroxysuccinimide active ester having a spacer represented by the formula:

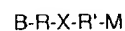


wherein X is a substituted or unsubstituted amino acid having from three to ten carbon atoms in a straight chain;  
n is from one to ten; and  
R is an alkyl, cycloalkyl, alkyl-cycloalkyl or an aromatic carboxylic ring.

28. The method according to Claim 25, wherein at least one coupling agent is an active halogen-N-hydroxysuccinimide active ester selected from the group consisting of N-succinimidyl bromoacetate, N-succinimidyl (4-iodoacetyl)aminobenzoate and sulfosuccinimidyl (4-iodoacetyl)aminobenzoate.

#### Patentansprüche

1. Reaktive Festphase, die ein immobilisiertes spezifisch bindendes Glied aufweist, umfassend eine Verbindung nach folgender Formel:



wobei B eine feste Phase ist, die ein reaktives Glied aufweist, das aus der Gruppe gewählt ist, die aus Amino-, Carboxyl- und Thiolgruppen

besteht;  
 worin M ein spezifisch bindendes Glied ist, das eine Aminogruppe aufweist;  
 worin R und R' Kupplungsagenzien sind, die unabhängig aus der Gruppe gewählt sind, die aus heterobifunktionellen und homobifunktionellen Reagenzien besteht; und  
 worin X eine Dithioverbindung ist, die an R über einen Thioether und an R' über einen Thioether gebunden ist; und worin R an B über die Amino-, Carboxyl- oder Thiolgruppe von B gebunden ist, und worin R' an M über die Aminogruppe von M gebunden ist.

2. Reaktive Festphase, die ein reaktives Thiol aufweist, umfassend eine Verbindung nach folgender Formel:



worin B eine feste Phase ist, die ein reaktives Glied aufweist, das aus der Gruppe gewählt ist, die aus Amino-, Carboxyl- und Thiolgruppen besteht;  
 worin R ein Kupplungsagens ist, das aus der Gruppe gewählt ist, die aus heterobifunktionellen und homobifunktionellen Reagenzien besteht; und  
 worin XH eine Dithioverbindung ist, die an R gebunden ist, worin eine Thiofunktion einen Thioether mit R ausbildet und worin die andere Thiofunktion das reaktive Thiol ist.

3. Reaktive Festphase nach Anspruch 1 oder 2, worin die feste Phase aus der Gruppe gewählt ist, die aus polymeren Perlen, Mikropartikeln, Röhrchen, Bögen, Platten, Objektträgern, Vertiefungen und Bändern gewählt ist.  
 4. Reaktives Festphasenglied nach Anspruch 1, worin R und R' heterobifunktionelle Kupplungsagenzien sind, die beide gleich sind.  
 5. Immobilisiertes spezifisch bindendes Glied nach Anspruch 1, worin das spezifisch bindende Glied ein Glied eines immunreaktiven spezifischen Bindungspaares ist.  
 6. Reaktives Festphasenglied nach Anspruch 1 oder 2, das des weiteren ein Trägermedium umfaßt.  
 7. Immobilisiertes spezifisch bindendes Glied nach Anspruch 1, worin die feste Phase eine polymere Mikropartikel ist, und worin das spezifisch bindende Glied ein Antigen ist.  
 8. Immobilisiertes spezifisch bindendes Glied nach

Anspruch 7, worin die feste Phase eine Aminomikropartikel ist, und worin das spezifisch bindende Glied ein HIV-Antigen ist.

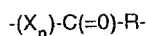
9. Immobilisiertes spezifisch bindendes Glied nach Anspruch 1, wobei das spezifisch bindende Glied aus der Gruppe gewählt ist, die aus einem Polynukleotid und einem Polypeptid besteht.  
 10. Reaktives Festphasenglied nach Anspruch 1 oder 2, worin das/die Kupplungsagenz/ien unabhängig aus der Gruppe gewählt ist/sind, die aus Maleimido-N-hydroxysuccinimid-Aktivestern und aus aktiven Halogen-N-hydroxysuccinimid-Aktivestern besteht.  
 11. Reaktive Festphase nach Anspruch 10, worin wenigstens ein Kupplungsagens ein Maleimido-N-hydroxysuccinimidester ist, der aus der Gruppe gewählt ist, die aus *m*-Maleimidobenzoyl-N-hydroxysuccinimidester, Succinimidyl-4-(N-maleimidomethyl)-cyclohexan-1-carboxylat, Succinimidyl-4-(*p*-maleimidophenyl)-butyrat, *m*-Maleimidobenzoylsulfosuccinimidester und aus Sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrat gewählt ist.  
 12. Reaktive Festphase nach Anspruch 10, worin wenigstens ein Kupplungsagens ein Maleimido-N-hydroxysuccinimid-Aktivester ist, der einen Spacer aufweist, der durch folgende Formel dargestellt wird:  

$$-(X_n)-C(=O)-R-$$

worin X eine substituierte oder unsubstituierte Aminosäure mit drei bis zehn Kohlenstoffatomen in gerader Kette ist;  
 worin n von eins bis zehn läuft; und  
 worin R gleich Alkyl, Cycloalkyl, Alkylcycloalkyl oder ein aromatischer carboxylischer Ring ist.

 13. Immobilisiertes spezifisch bindendes Glied nach Anspruch 10, worin wenigstens ein Kupplungsagens ein aktiver Halogen-N-hydroxysuccinimid-Aktivester ist, der aus der Gruppe gewählt ist, die aus folgendem besteht: N-Succinimidylbromacetat, N-succinimidyl-(4-jodacetyl)aminobenzoat und Sulfosuccinimidyl-(4-jodacetyl)aminobenzoat.  
 14. Verfahren zur Herstellung eines immobilisierten spezifisch bindenden Gliedes, das folgende Schritte umfaßt:  
 a. Umsetzung einer festen Phase, die ein reaktives Glied aufweist, das aus der Gruppe gewählt ist, die aus Amino-, Carboxyl- und Thiolgruppen besteht, mit einem ersten Kupp-

- lungensagens, das aus der Gruppe gewählt ist, die aus heterobifunktionellen und homobifunktionellen Reagenzien besteht, wobei das erste Kupplungsagens eine erste Funktion aufweist, die gegenüber dem reaktiven Glied der festen Phase reaktiv ist, und eine zweite Funktion, die gegenüber einem Thiol reaktiv ist, wobei ein feste Phase/Kupplungsagens-Komplex durch die Reaktion zwischen dem reaktiven Glied und der ersten Funktion des ersten Kupplungsagenses ausgebildet wird;
- b. Umsetzen eines spezifisch bindenden Gliedes, das eine Aminogruppe aufweist, mit einem zweiten Kupplungsagens, das eine erste Funktion aufweist, die gegenüber der Aminogruppe reaktiv ist, und eine zweite Funktion, die gegenüber einem Thiol reaktiv ist, wodurch ein spezifisch bindendes Glied/Kupplungsagens-Komplex ausgebildet wird, durch die Reaktion zwischen der Aminogruppe und der ersten Funktion des zweiten Kupplungsagenses; und
- c. Umsetzen des feste Phase/Kupplungsagens-Komplexes und des spezifisch bindenden Glied/Kupplungsagens-Komplexes mit einer Dithiolverbindung, wobei ein feste Phase/spezifisch bindendes Glied-Komplex ausgebildet wird, in dem die feste Phase und das spezifisch bindende Glied über zwei Thioether miteinander gekuppelt sind.
15. Verfahren zur Herstellung einer thiolierten festen Phase, das folgende Schritte umfaßt:
- a. Umsetzen einer festen Phase, die ein reaktives Glied aufweist, das aus der Gruppe gewählt ist, die aus Amino-, Carboxyl- und Thiolgruppen besteht, mit einem Kupplungsagens, das aus der Gruppe gewählt ist, die aus heterobifunktionellen und homobifunktionellen Reagenzien besteht, wobei ein feste Phase/Kupplungsagens-Komplex ausgebildet wird; und
- b. Umsetzen des feste Phase/Kupplungsagens-Komplexes mit einer Dithiolverbindung, worin eine Thiolgruppe die Dithiolverbindung an den feste Phase/Kupplungsagens-Komplex über einen Thioether bindet, und worin die andere Thiolgruppe das reaktive Thiol darstellt.
16. Verfahren nach Anspruch 14 oder 15, worin die feste Phase aus der Gruppe gewählt ist, die aus polymeren Perlen, Mikropartikeln, Röhrchen, Bögen, Platten, Objektträgern, Vertiefungen und Bändern besteht.
17. Verfahren nach Anspruch 14 oder 15, worin das/die Kupplungsagens/ien (ein) heterobifunktionelle/s Reagenz/ien ist/sind.
18. Verfahren nach Anspruch 14, das des weiteren den Schritt des Einbaus des kreuzvernetzten feste Phase/spezifisch bindendes Glied-Komplexes auf einem oder innerhalb eines Trägermediums umfaßt.
19. Verfahren nach Anspruch 14, worin das spezifisch bindende Glied ein Glied eines immunreaktiven spezifischen Bindungspaares ist.
20. Verfahren nach Anspruch 19, worin die feste Phase eine polymere Mikropartikel ist, und worin das spezifisch bindende Glied ein Antigen ist.
21. Verfahren nach Anspruch 20, worin die feste Phase eine Aminomikropartikel ist, und worin das spezifisch bindende Glied ein HIV-Antigen ist.
22. Verfahren nach Anspruch 14, worin das spezifisch bindende Glied aus der Gruppe gewählt ist, die aus einem Polynukleotid und einem Polypeptid besteht.
23. Verfahren nach Anspruch 14 worin der feste Phase/Kupplungsagens-Komplex mit der Dithiolverbindung unter Ausbildung eines thiolierten feste Phase/Kupplungsagens-Komplexes umgesetzt wird, und wobei dann der spezifisch bindendes Glied/Kupplungsagens-Komplex mit dem thiolierten feste Phase/Kupplungsagens-Komplex umgesetzt wird.
24. Verfahren nach Anspruch 14, worin der spezifisch bindendes Glied/Kupplungsagens-Komplex mit der Dithiolverbindung unter Ausbildung eines spezifisch bindendes Glied/Kupplungsagens-Komplex umgesetzt wird, und worin dann der feste Phase/Kupplungsagens-Komplex mit dem thiolierten spezifisch bindendes Glied/Kupplungsagens-Komplex umgesetzt wird.
25. Verfahren nach Anspruch 14 oder 15, worin das Kupplungsagens/ien unabhängig aus der Gruppe gewählt ist/sind, die aus Maleimido-N-hydroxysuccinimid-Aktivestern und aktiven Halogen-N-hydroxysuccinimid-Aktivestern besteht.
26. Verfahren nach Anspruch 25, worin wenigstens ein Kupplungsagens ein Maleimido-N-hydroxysuccinimidester ist, der aus der Gruppe gewählt ist, die aus folgendem besteht: *m*-Maleimidobenzoyl-N-hydroxysuccinimidester; Succinimidyl-4-(N-maleimidomethyl)cyclohexan-1-carboxylat; Succinimidyl-4-(*p*-maleimidophenyl)butyrat; *m*-Maleimidobenzoylsulfosuccinimidester und Sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrat. \*\*
27. Verfahren nach Anspruch 25, worin wenigstens ein Kupplungsagens ein Maleimido-N-hydroxysuccinimid-Aktivester mit einem Spacer ist, der durch folgende Formel dargestellt wird:

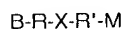


worin X eine substituierte oder unsubstituierte Aminosäure mit drei bis zehn Kohlenstoffatomen in gerader Kette ist;  
 worin n von eins bis zehn läuft; und  
 worin R gleich Alkyl, Cycloalkyl, Alkyl-cycloalkyl oder ein aromatischer carboxylischer Ring ist.

28. Verfahren gemäß Anspruch 25, worin wenigstens ein Kupplungsagens ein aktiver Halogen-N-hydroxysuccinimid-Aktivester ist, der aus der Gruppe gewählt ist, die aus N-Succinimidylbromacetat, N-Succinimidyl-(4-jodacetyl)aminobenzoat und Sulfo-succinimidyl-(4-jodacetyl)aminobenzoat besteht.

#### Revendications

1. Phase solide réactive, comportant un membre immobilisé d'une liaison spécifique, comprenant un composé de formule :



dans laquelle B représente une phase solide comportant un membre réactif choisi dans l'ensemble consistant en des groupes amino, carboxyle et thiol ;

M est un membre d'une liaison spécifique comportant un groupe amino ;

R et R' sont des agents de couplage, choisis, indépendamment l'un de l'autre, dans l'ensemble consistant en des réactifs hétérobifonctionnels et des réactifs homobifonctionnels; et

X est un composé de type dithio relié à R par l'intermédiaire d'un thioéther et relié à R' par l'intermédiaire d'un thioéther, R étant fixé sur B par l'intermédiaire du groupe amino, carboxyle ou thiol de B et R' étant fixé sur M par l'intermédiaire du groupe amino de M.

2. Phase solide réactive, ayant un thiol réactif, comprenant un composé de formule :



dans laquelle B représente une phase solide comportant un membre réactif choisi dans l'ensemble consistant en des groupes amino, carboxyle et thiol ;

R est un agent de couplage, choisi dans l'ensemble consistant en des réactifs hétérobifonctionnels et des réactifs homobifonctionnels ; et

XH est un composé de type dithio relié à R, l'une des fonctions thio formant un thioéther avec R et l'autre fonction thio constituant le thiol réactif.

3. Phase solide réactive selon la revendication 1 ou 2, dans laquelle ladite phase solide est choisie dans l'ensemble consistant en des perles polymères, des microparticules, des tubes, des feuilles, des plaques, des lames, des puits et des rubans.

4. Membre d'une phase solide réactive selon la revendication 1, dans lequel R et R' représentent des agents hétérobifonctionnels de couplage, qui sont les mêmes tous les deux.

5. Membre immobilisé d'une liaison spécifique selon la revendication 1, dans lequel ledit membre de liaison spécifique est un membre d'une paire capable d'une liaison spécifique par immunoréaction.

6. Membre réactif d'une phase solide selon la revendication 1 ou 2, comprenant en outre un milieu formant support.

7. Membre immobilisé d'une liaison spécifique selon la revendication 1, dans lequel ladite phase solide est une microparticule polymère et ledit membre de liaison spécifique est un antigène.

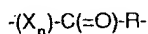
8. Membre immobilisé d'une liaison spécifique selon la revendication 7, dans lequel ladite phase solide est une microparticule aminée et ledit membre de liaison spécifique est un antigène de HIV.

9. Membre immobilisé d'une liaison spécifique selon la revendication 1, dans lequel ledit membre de liaison spécifique est choisi dans l'ensemble consistant en un polynucléotide et un polypeptide.

10. Membre d'une phase solide réactive selon la revendication 1 ou 2, dans lequel ledit ou lesdits agents de couplage sont choisis, indépendamment l'un de l'autre, dans l'ensemble consistant en des esters actifs de maléimido-N-hydroxysuccinimide et des esters actifs d'un halogéno(actif)-N-hydroxysuccinimide.

11. Phase solide réactive selon la revendication 10, dans laquelle au moins un agent de couplage est un ester de maléimido-N-hydroxysuccinimide choisi dans l'ensemble consistant en l'ester de m-maléimidobenzoyl-N-hydroxysuccinimide, le 4-(N-maléimidométhyl)cyclohexane-1-carboxylate de succinimidyle, le 4-(p-maléimidophényl)butyrate de succinimidyle ; l'ester de m-maléimidobenzoyl-sulfosuccinimide et le 4-(p-maléimidophényl)butyrate de sulfosuccinimidyle.

12. Phase solide réactive selon la revendication 10, dans laquelle au moins un agent de couplage est un ester actif de maléimido-N-hydroxysuccinimide ayant un espaceur représenté par la formule :



dans laquelle X représente un acide aminé, substitué ou non substitué, comportant de 3 à 10 atomes de carbone en chaîne linéaire ; n vaut 1 à 10 ; et R représente un groupe alkyle, cycloalkyle, alkyl-cycloalkyle ou un noyau carboxylique aromatique.

13. Membre immobilisé d'une liaison spécifique selon la revendication 10, dans lequel au moins un agent de couplage est un ester actif d'halogéno(actif)-N-hydroxysuccinimide choisi dans l'ensemble consistant en le bromoacétate de N-succinimidyle, le (4-iodoacétyl)aminobenzoate de N-succinimidyle et le (4-iodoacétyl)aminobenzoate de sulfosuccinimidyle.

14. Procédé pour produire un membre immobilisé d'une liaison spécifique, comprenant les étapes consistant à :

a. faire réagir une phase solide, comportant un membre réactif choisi dans l'ensemble consistant en des groupes amino, carboxyle et thiol, avec un premier agent de couplage choisi dans l'ensemble consistant en des réactifs hétérobifonctionnels et des réactifs homobifonctionnels ayant un premier groupe fonctionnel pouvant réagir avec le membre réactif de ladite phase solide et un second groupe fonctionnel pouvant réagir avec un thiol, en formant ainsi un complexe phase solide/agent de couplage par réaction entre ledit membre réactif et le premier groupe fonctionnel du premier agent de couplage ;  
b. faire réagir un membre d'une liaison spécifique, ayant un groupe amino, avec un second agent de couplage comportant un premier groupe fonctionnel pouvant réagir avec ledit groupe amino et un second groupe fonctionnel pouvant réagir avec un thiol, en formant ainsi un complexe membre d'une liaison spécifique/agent de couplage par réaction entre ledit groupe amino et le premier groupe fonctionnel du second agent de couplage ; et  
c. faire réagir ledit complexe phase solide/agent de couplage et ledit complexe membre d'une liaison spécifique/agent de couplage avec un dithiol, en formant ainsi un complexe phase solide/membre d'une liaison spécifique

dans lequel la phase solide et le membre d'une liaison spécifique sont couplés ensemble par l'intermédiaire de deux thioéthers.

- 5 15. Procédé pour produire une phase solide thiolée, comprenant les étapes consistant à :

a. faire réagir une phase solide, comportant un membre réactif choisi dans l'ensemble consistant en des groupes amino, carboxyle et thiol, avec un agent de couplage choisi dans l'ensemble consistant en des réactifs hétérobifonctionnels et des réactifs homobifonctionnels, en formant ainsi un complexe phase solide/agent de couplage ; et  
b. faire réagir ledit complexe phase solide/agent de couplage avec un dithiol, réaction dans laquelle un groupe thiol relie le dithiol audit complexe phase solide/agent de couplage par l'intermédiaire d'un thioéther et l'autre groupe thiol est le thiol réactif.

16. Procédé selon la revendication 14 ou 15, dans lequel ladite phase solide est choisie dans l'ensemble consistant en des perles polymères, des microparticules, des tubes, des feuilles, des plaques, des lames, des puits et des rubans.

17. Procédé selon la revendication 14 ou 15, dans lequel ledit ou lesdits agents de couplage sont des réactifs hétérobifonctionnels.

18. Procédé selon la revendication 14, comprenant en outre l'incorporation dudit complexe phase solide/membre d'une liaison spécifique, réticulé, sur ou dans un milieu formant support.

19. Procédé selon la revendication 14, dans lequel ledit membre d'une liaison spécifique est un membre d'une paire de liaison spécifique par immunoréaction.

20. Procédé selon la revendication 19, dans lequel ladite phase solide est une microparticule polymère et ledit membre d'une liaison spécifique est un antigène.

21. Procédé selon la revendication 20, dans lequel ladite phase solide est une microparticule aminée et ledit membre d'une liaison spécifique est un antigène de HIV.

22. Procédé selon la revendication 14, dans lequel ledit membre d'une liaison spécifique est choisi dans l'ensemble consistant en un polynucléotide et un polypeptide.

23. Procédé selon la revendication 14, dans lequel ledit

complexe phase solide/agent de couplage est mis en réaction avec le dithiol pour former un complexe thiolé phase solide/agent de couplage puis le complexe membre d'une liaison spécifique/agent de couplage est mis en réaction avec ledit complexe thiolé phase solide/agent de couplage. 5

24. Procédé selon la revendication 14, dans lequel ledit complexe membre d'une liaison spécifique/agent de couplage est mis en réaction avec le dithiol pour former un complexe thiolé membre d'une liaison spécifique/agent de couplage, puis le complexe phase solide/agent de couplage est mis en réaction avec ledit complexe thiolé membre d'une liaison spécifique/agent de couplage. 10 15

25. Procédé selon la revendication 14 ou 15, dans lequel ledit ou lesdits agents de couplage sont choisis, indépendamment, dans l'ensemble consistant en des esters actifs de maléimido-N-hydroxysuccinimide et des esters actifs d'halogéno(actif)-N-hydroxysuccinimide. 20

26. Procédé selon la revendication 25, dans lequel au moins un agent de couplage est un ester de maléimido-N-hydroxysuccinimide choisi dans l'ensemble consistant en l'ester de m-maléimidobenzoyl-N-hydroxy-succinimide, le 4-(N-maléimidométhyl)cyclohexane-1-carboxylate de succinimidyle, le 4-(p-maléimidophényl)butyrate de succinimidyle, l'ester de m-maléimidobenzoyl-sulfosuccinimide et le 4-(p-maléimidophényl)butyrate de sulfosuccinimidyle. 25 30

27. Procédé selon la revendication 25, dans lequel au moins un agent de couplage est un ester actif de maléimido-N-hydroxysuccinimide ayant un espaceur représenté par la formule : 35



dans laquelle X représente un aminoacide substitué ou non substitué comportant de 3 à 10 atomes de carbone en chaîne linéaire, n vaut de 1 à 10 ; et 45

R représente un groupe alkyle, cycloalkyle, alkyl-cycloalkyle ou un noyau carboxylique aromatique.

28. Procédé selon la revendication 25, dans lequel au moins un agent de couplage est un ester actif d'halogéno(actif)-N-hydroxysuccinimide choisi dans l'ensemble consistant en le bromoacétate de N-succinimidyle, le (4-iodoacétyl)aminobenzoate de N-succinimidyle et le (4-iodoacétyl)amino-benzoate de sulfosuccinimidyle. 50 55



Activation of a Specific Binding Member Using a Maleimido-NHS Active Ester Heterobifunctional Reagent

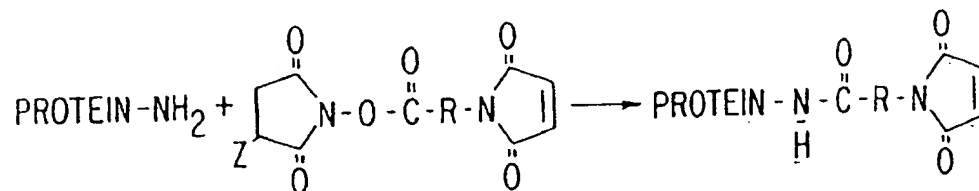


FIG. 1

Activation of a Specific Binding Member Using an Active Halogen-NHS Active Ester Heterobifunctional Reagent

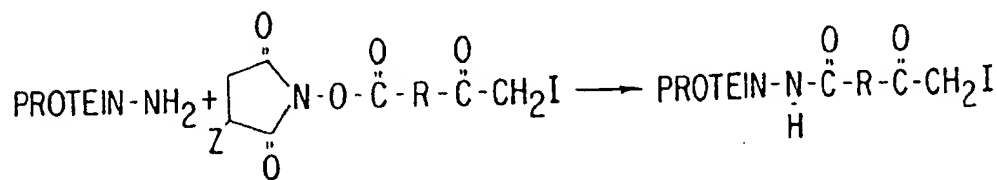


FIG. 2

## Dithiol Preparation of a Thiolated Solid Phase

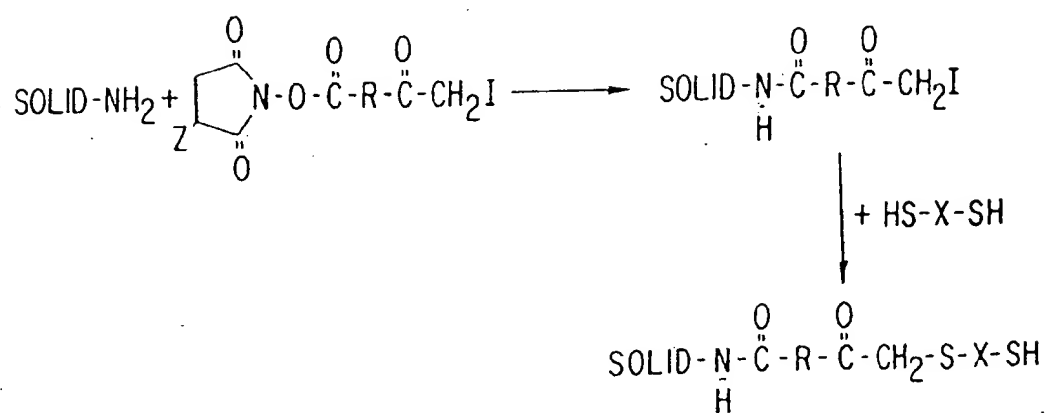


FIG. 3

## Preparation of a Thiolated Solid Phase and Covalent Immobilization of a Specific Binding Member

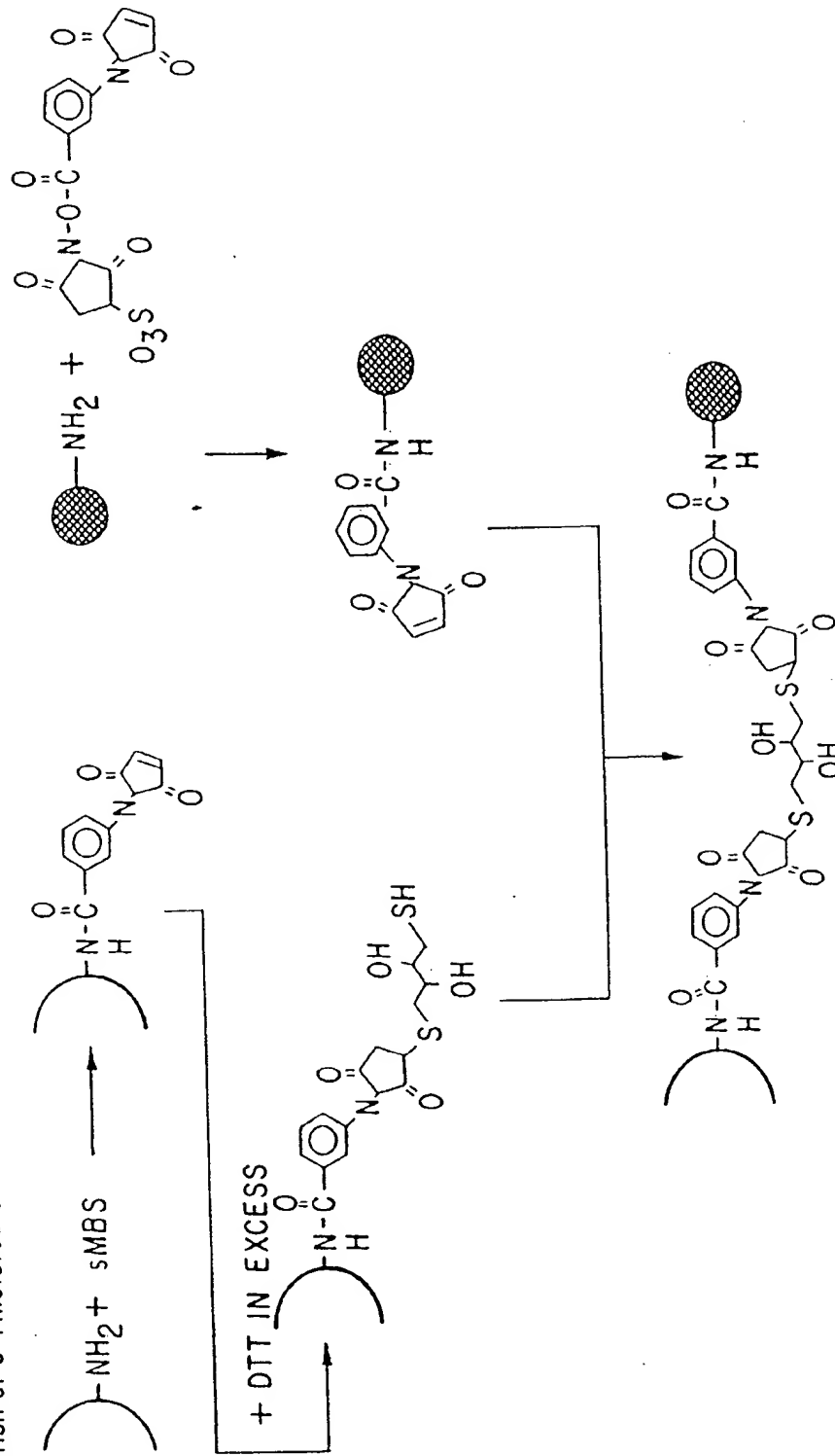


FIG. 4